

CENTRAL TOLERANCE: LEARNING SELF-CONTROL IN THE THYMUS

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Abstract | In the past few years, there has been a flurry of discoveries and advancements in our understanding of how the thymus prepares T cells to exist at peace in normal healthy tissue: that is, to be self-tolerant. In the thymus, one of the main mechanisms of T-cell central tolerance is clonal deletion, although the selection of regulatory T cells is also important and is gaining enormous interest. In this Review, we discuss the emerging consensus about which models of clonal deletion are most physiological, and we review recent data that define the molecular mechanisms of central tolerance.

ANERGY

A state of non-responsiveness to antigen. Anergic T or B cells cannot respond to their cognate antigens under optimal conditions of stimulation.

RECEPTOR EDITING

A tolerance mechanism in which the binding of self-antigen during development promotes secondary antigen-receptor gene rearrangement. In the T-cell receptor α -chain or immunoglobulin light chain locus, this results in replacement of the autoreactive receptor with a benign one. This is thought to be one of the main tolerance mechanisms in B-cell development, but it is less well-described for T cells.

Central tolerance refers to those events in the early life of a lymphocyte that focus the adaptive immune system on pathogens and steer it away from healthy tissue. It is induced at the primary sites of lymphocyte development — the bone marrow for a developing B cell and the thymus for a developing T cell — and it encompasses all of the mechanisms by which antigen-receptor recognition of self-antigen at these sites results in self-tolerance. Although central-tolerance mechanisms are efficient, they cannot eliminate all self-reactive lymphocytes, in part because not all self-antigens are expressed at the primary site of lymphocyte development. Therefore, peripheral-tolerance mechanisms exist, and these induce lymphocytes that first encounter their cognate self-antigen outside the primary site of development to become tolerant.

For developing T cells, many of the randomly rearranged antigen receptors are useless, because these receptors cannot bind the MHC alleles that are present in the individual. So, positive selection is a crucial step that enriches for T-cell progenitors that are MHC restricted by allowing only cells that express a T-cell receptor (TCR) that can interact with self-peptide-MHC complexes to differentiate further. This step, of course, also enriches for self-reactive cells, thereby making the danger of autoimmunity inherent in the adaptive immune system. Fortunately, the most strongly self-reactive progenitors are under strict control — that is, they are made self-tolerant — and it is the weakly reactive progenitors that

mature, populate the lymphoid organs and participate in immune responses to foreign antigens (FIG. 1).

The hallmark of T-cell central tolerance is clonal deletion: that is, suicide of T-cell progenitors that have high affinity for self-antigens¹. Other processes have been described, including ANERGY² and RECEPTOR EDITING^{3,4}, but these are thought to have a lesser role. These three processes impair or eliminate high-affinity self-reactive cells and are considered to be negative-selection mechanisms (FIG. 1). But not all central-tolerance mechanisms cripple self-reactive T cells. The positive selection of regulatory T-cell populations in the thymus enables T cells to actively restrain immune responses to motifs that are recognized as self (FIG. 1). Three main cell types have been considered as potential regulatory T-cell subsets: CD4⁺CD25⁺ REGULATORY T (T_{reg}) CELLS^{5,6}, CD8 $\alpha\alpha$ ⁺ INTESTINAL EPITHELIAL LYMPHOCYTES⁷ and NATURAL KILLER T (NKT) CELLS⁸. All are thought to be induced by high affinity (that is, agonist) self-peptide-MHC interactions with TCR in the thymus⁹. The double whammy of 'recessive' (that is, cell intrinsic) and 'dominant' (that is, *trans*-acting) central-tolerance mechanisms greatly reduces the threat of autoimmunity inherent in the adaptive immune system. Indeed, the significance of these processes to immune health has been directly shown in recent years as the molecular basis of two inherited autoimmune syndromes has been uncovered. In both cases, the affected molecules are transcriptional regulators that are crucial for these two distinct central-tolerance

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mechanisms: mutations in the autoimmune regulator (*AIRE*) gene lead to defective clonal deletion of T cells and to the multi-organ syndrome known as autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy syndrome (APECED)¹⁰, whereas mutations in the fork-head box P3 (*FOXP3*) gene impair the development of T_{Reg} cells and cause the syndrome known as immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome (IPEX)⁶. Therefore, although there are many peripheral mechanisms that control lymphocyte reactivity, central-tolerance mechanisms, which operate during development, seem to be essential for the maintenance of self-tolerance, and it is vital that we understand these cellular and molecular processes in detail. Here, we provide an overview of our current knowledge of the mechanisms that regulate central tolerance, and we highlight areas that require further investigation.

CD4⁺CD25⁺ REGULATORY T CELL

(T_{Reg} cell). A CD4⁺ T cell that expresses high levels of CD25 (also known as the α -chain of the interleukin-2 receptor), is naturally anergic and requires stimulation through the T-cell receptor for induction of its cell-mediated suppressive function. The role of this subset of T cells is to maintain self-tolerance.

CD8 $\alpha\alpha$ ⁺ INTESTINAL EPITHELIAL LYMPHOCYTE

A type of T cell that is found in the intestinal epithelium. The CD8 molecule that they express is a homodimer of CD8 α , rather than the CD8 $\alpha\beta$ heterodimer that is expressed by conventional CD8⁺ T cells in the lymph nodes. It has been proposed that these cells are self-reactive T cells that have regulatory properties.

NATURAL KILLER T CELL

(NKT cell). A T cell that expresses both natural killer (NK)-cell receptors and an $\alpha\beta$ -T-cell receptor ($\alpha\beta$ -TCR). In mice, these cells were first identified by their expression of the alloantigen NK1.1 (also known as NKR-P1C). Some mouse NKT cells express an invariant TCR that uses the V α 14 variable region of the TCR α -chain and recognizes CD1d-associated antigen. NKT cells are characterized by cytolytic activity and rapid production of cytokines, including interferon- γ and interleukin-4, and they might regulate the function of other T cells.

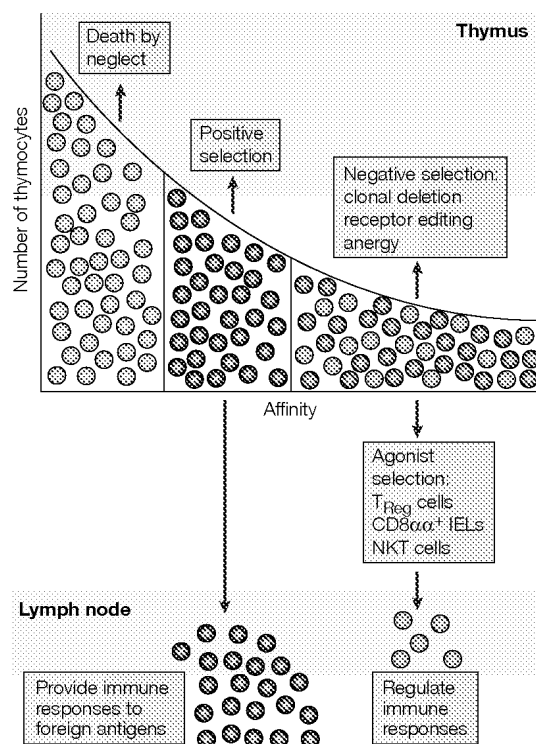


Figure 1 | Central-tolerance mechanisms. The affinity of the T-cell receptor (TCR) for self-peptide-MHC ligands is the crucial parameter that drives developmental outcome in the thymus. Progenitors that have no affinity or very low affinity die by neglect. This is thought to be the fate of most thymocytes. If the TCR has a low affinity for self-peptide-MHC, then the progenitor survives and differentiates, a process that is known as positive selection. If the progenitor has a high affinity for self-peptide-MHC, then several outcomes are possible. First, the progenitor can be selected against, a process that is known as negative selection. The main mechanism of negative selection is clonal deletion, but receptor editing and anergy have also been described. Second, there seem to be mechanisms that select for high-affinity self-reactive cells and result in differentiation into a 'regulatory'-cell phenotype. It is not known what determines whether a T cell is tolerated by negative selection or is selected to become a regulatory T cell⁹. IEL, intestinal epithelial lymphocyte; NKT cell, natural killer T cell; T_{Reg} cell, CD4⁺CD25⁺ regulatory T cell.

The model of clonal deletion matters

Despite many years of study, the molecular pathway of clonal deletion remains enigmatic. The antigen receptor is, of course, crucial, but how signals from this receptor induce the expression of suicide genes and how those genes regulate apoptosis is not clear¹. In part, this might be a consequence of the lack of consensus about the best way to experimentally model clonal deletion. Early on, investigators injected crosslinking antibody that is specific for the TCR-CD3 complex into mice, and they observed thymocyte death. Although convenient, this approach induces massive peripheral T-cell activation, which elicits the production of stress hormones and cytokines that contribute to the death of thymocytes non-specifically. Indeed, it was recently shown that the glucocorticoid receptor is essential for the death of thymocytes in this model of clonal deletion¹¹. A similar phenomenon probably occurs in TCR-transgenic mice that are injected with specific peptide. Martin and Bevan¹² showed that adoptive transfer of small numbers of mature TCR-transgenic T cells into non-transgenic recipients followed by injection with cognate antigen led to the death of endogenous thymocytes that were not specific for that antigen. For these and other reasons, many investigators have favoured the use of TCR-transgenic mice that co-express the cognate antigen of the TCR as an endogenous self-antigen for the modelling of negative selection of thymocytes by clonal deletion.

A classic model of this type is the H-Y model, in which CD8⁺ T cells that express the H-Y TCR, which is specific for the male antigen H-Y, are positively selected in female mice but deleted in male mice¹³. TCR-transgenic mice tend to fall into two categories of clonal deletion: those that delete thymocytes early in development (that is, at the double negative (DN) to double positive (DP) stage); and those that delete thymocytes late (that is, at the DP to single positive (SP) stage). Sometimes these are referred to as cortical and medullary deletion, respectively, because DN and DP progenitors reside in the cortex (which is the outer area of the thymus) and SP progenitors reside in the medulla (which is the inner area). Of the more than 50 TCR-transgenic mice that have been described so far, 32 have been used to model clonal deletion. In about half of these, thymocytes are deleted early in development, and in about half, thymocytes are deleted late in development. (For a complete list of TCR-transgenic strains, see Models of negative selection in the Online links box.) One determinant of early clonal deletion versus late clonal deletion is whether the self-antigen is present in both the cortex and the medulla or is present only in the medulla; in the latter case, deletion occurs at a late stage, because progenitors do not migrate to the medulla until late in development. However, even for antigens that are broadly expressed throughout the thymus, deletion occurs early in some models and late in others, and evidence indicates that one of the factors that can contribute to the timing of thymocyte deletion in TCR-transgenic mice is the affinity of the TCR for its cognate ligand¹⁴. Nonetheless, many investigators

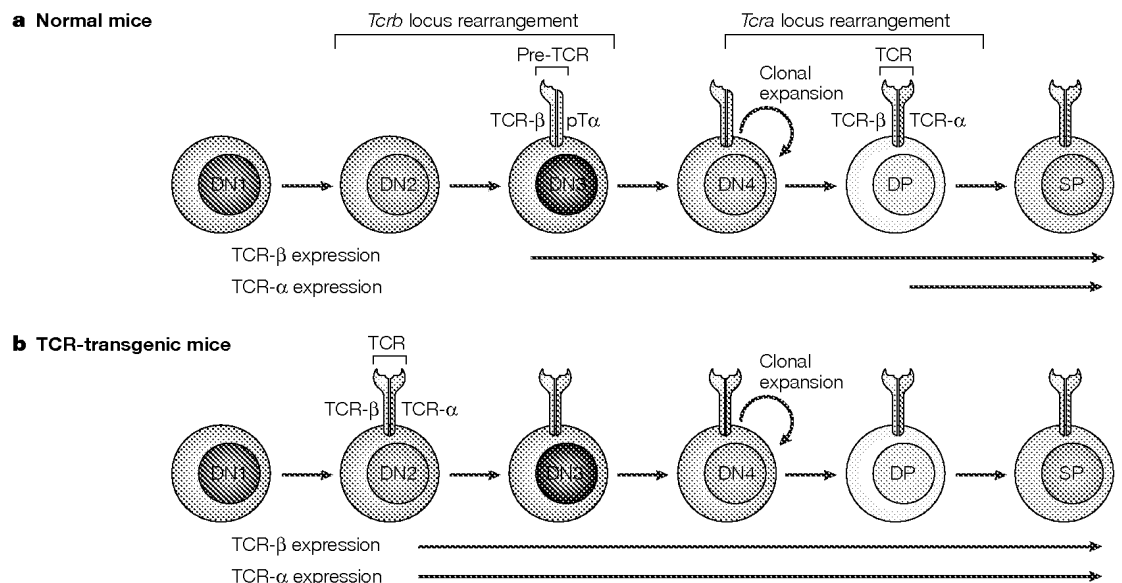


Figure 2 | TCR-transgenic mice express a cell-surface TCR earlier in development than do normal mice. a As progenitors develop in the thymus, they express the recombination-activating genes (RAGs) and undergo rearrangement at the T-cell receptor β -chain (*Tcrb*) locus during the double negative 2 (DN2) or DN3 stage. The chromatin configuration of the *Tcrb* locus is accessible at this stage. When a productive TCR β -chain is created, it pairs with the invariant pre-TCR α -chain (pT α), forming a pre-TCR that transduces a signal for further differentiation towards the DN4 and double positive (DP) stages. During this transition, the *Tcrb* locus becomes inaccessible, and the progenitor undergoes several rounds of division during which RAG1 and RAG2 are not active. As the cell progresses to the DP stage, RAG1 and RAG2 become active again; the *Tcrα* locus also changes to an accessible chromatin configuration, and it now undergoes rearrangement. This temporal segregation of *Tcrb* and *Tcrα* rearrangement means that an intact $\alpha\beta$ -TCR heterodimer is not produced until the DP stage. **b** By contrast, in TCR-transgenic animals, both chains of the $\alpha\beta$ -TCR heterodimer are expressed early in development (typically at the DN stage, but this can vary slightly depending on the promoters and/or enhancers that are used). Interestingly, even in 'knock-in' mice in which a rearranged VJ (variable region-joining region) is in the appropriate location in the chromosome, expression is observed early, at the DN stage. This creates a potential problem for using TCR-transgenic models to study selection, because the ligation of the $\alpha\beta$ -TCR heterodimer might have different (non-physiological) consequences when it is expressed at the DN stage. SP, single positive.

Cre-loxP TECHNOLOGY

A site-specific recombination system. Two short DNA sequences (loxP sites) are engineered to flank the target DNA. Expression of the recombinase Cre leads to excision of the intervening sequence. Depending on the type of promoter that controls Cre expression, Cre can be expressed at specific times during development or by specific subsets of cells.

Lck-Cre MOUSE

A mouse that expresses the recombinase Cre under the control of the *Lck* proximal promoter. Cre expression is initiated early during thymic development, at the double-negative-2 stage or earlier.

ENDOGENOUS SUPERANTIGEN

A defective mammary tumour-virus sequence that is stably integrated in the genome of certain mouse strains. This sequence encodes an MHC-class-II-binding protein that causes stimulation of T cells that use specific variable regions in the T-cell receptor β -chain.

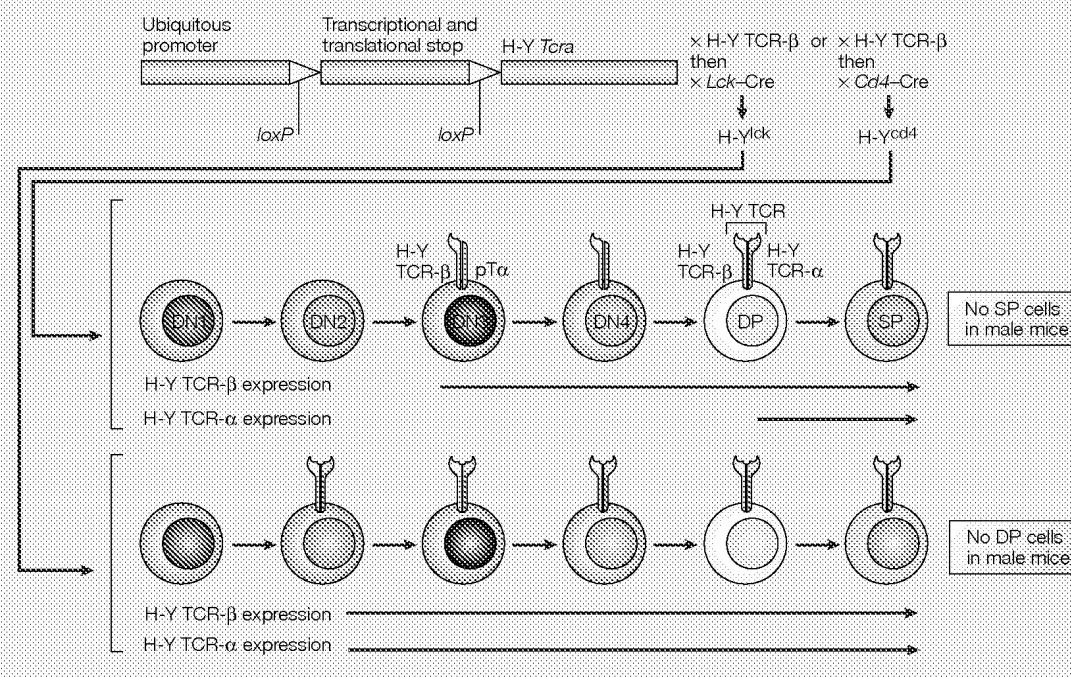
have questioned whether early clonal deletion occurs in normal animals, because the temporal separation of rearrangement of the *TCRA* and *TCRB* genes (which encode the α -chain and β -chain of the TCR, respectively) dictates that antigen receptors are not formed until the DP stage of thymocyte development (FIG. 2).

Our research group¹⁵ recently tested this directly, using Cre-loxP TECHNOLOGY to create a transgenic animal that mimics the normal timing of TCR expression (BOX 1). We used transgenic mice expressing the β -chain of the H-Y TCR and crossed them with transgenic mice expressing the α -chain of the H-Y TCR, expression of which was controlled by expression of the recombinase Cre. When these mice were crossed with *Lck*-Cre MICE, both H-Y TCR- α and H-Y TCR- β were expressed early in development, in DN thymocytes (similar to conventional H-Y-TCR-transgenic mice); these mice are denoted here as H-Y^{lck} mice. By contrast, when crossed with *Cd4*-Cre mice (in which expression of Cre is under the control of the *Cd4* promoter), H-Y TCR- α was not expressed until the DP stage (which is the stage at which TCR- α is expressed in normal mice); these mice are denoted here as H-Y^{cd4}. In both conventional H-Y-TCR transgenic mice and H-Y^{lck} mice, thymocyte deletion in male animals occurred at the DN stage, when the TCR was first expressed. However, in male H-Y^{cd4} mice, deletion did not occur at the DP stage, despite the

fact that DP thymocytes expressed the H-Y TCR and encountered H-Y antigen at this stage of development. Deletion in this more physiological model of T-cell development was not induced until late in development, at the SP stage¹⁵. Other new data also support the idea that early deletion is a non-physiological aspect of TCR-transgenic mice. Lew and colleagues¹⁶ used transgenic mice expressing a CD4⁺ T-cell-depleting antibody crossed with either DO11.10-TCR-transgenic mice or OT-II-TCR-transgenic mice (which express a TCR specific for an ovalbumin (OVA)-derived peptide in the context of I-A^d and I-A^b, respectively) to show that if peripheral CD4⁺ T cells were eliminated, then thymic clonal deletion occurred late in development instead of early in development. Furthermore, the SP (medullary) stage was indicated to be the stage of thymic clonal deletion of T cells in non-TCR-transgenic animals, as judged by the localization of apoptotic cells¹⁷ or the phenotype of cells expressing *Nur77*, a putative suicide gene¹⁸. Of course, clonal deletion of thymocytes at the SP stage was indicated from the first experimental description of clonal deletion, a landmark paper by the Kappler and Marrack group¹⁹. However, the source of antigen in that system was ENDOGENOUS SUPERANTIGEN, and it was possible that deletion in response to endogenous superantigens would be distinct from deletion in response to self-antigens.

Box 1 | Timing of TCR expression influences the stage at which clonal deletion occurs

To achieve the normal temporal separation of T-cell receptor (TCR) α -chain and β -chain expression (FIG. 2) for a defined TCR with a known cognate antigen, mice expressing the H-Y TCR β -chain were crossed with transgenic mice expressing the H-Y TCR α -chain in a Cre-dependent manner. Cre-dependent expression of H-Y TCR- α was achieved by inserting the H-Y *Tcr α* cDNA downstream of a transcriptional and translational stop cassette flanked by *loxP* sites. Following Cre-mediated removal of the stop cassette, a ubiquitous promoter (pCAGGS) can drive transcription of the H-Y *Tcr α* cDNA. When crossed with *Lck*-Cre mice (which express Cre under the control of the *Lck* promoter), the resultant mice, which are denoted here as H-Y^{Lck} mice, express both H-Y TCR- β and TCR- α in double negative (DN) thymocytes (similar to conventional H-Y-TCR-transgenic mice). By contrast, when crossed with *Cd4*-Cre mice (which express Cre under the control of the *Cd4* promoter), the resultant mice, which are denoted here as H-Y^{Cd4} mice, express H-Y TCR- β at the DN stage, but they do not express H-Y TCR- α until the double positive (DP) stage (similar to wild-type mice). Clonal deletion occurs early in development (at the DP stage) in H-Y^{Lck} mice, whereas it occurs late (at the CD8⁺ single positive (SP) stage) in H-Y^{Cd4} mice. These data indicate that clonal deletion normally occurs late in development and that the early deletion that has been observed for numerous TCR-transgenic mice is a consequence of ectopic TCR expression. pT α , pre-TCR α -chain.



It would seem that a consensus is now emerging in the field that clonal deletion normally occurs late in development (at the DP-to-SP transition). Although this might seem to be a minor detail, it provides an important conceptual framework for exploration of the molecular mechanism of thymic clonal deletion. First, the molecules that regulate cell survival and cell death might be substantially different at a late stage of thymocyte development compared with at an earlier stage of development. This idea is supported by the markedly different gene-expression profiles that are observed at these stages^{20–23}. So, we should not assume that the factors that are involved in apoptosis in early-deleting transgenic-mouse models are the same factors that are involved in clonal deletion in normal mice. Second, the accumulation of H-Y-reactive DP thymocytes in H-Y^{Cd4} mice (despite the presence of cognate antigen) implies that apoptosis is not immediately induced in self-reactive DP progenitors. This is an interesting mechanistic point, because it indicates that a simple differential TCR-signalling model might be insufficient to explain

the paradigm of positive selection versus negative selection (FIG. 3). It might imply that apoptosis requires the expression of additional genes: for example, upregulation of expression of a co-stimulatory receptor, signalling through which provides the developing thymocyte with a stimulus that induces cell death. Last, DN and DP thymocytes reside in different anatomical locations of the thymus than do SP thymocytes. So, it is possible that apoptosis requires upregulation of expression of a co-stimulatory receptor, signalling through which provides the developing thymocyte with a stimulus that induces cell death and for which the ligand is preferentially expressed in the medulla. For these reasons, it is timely to re-evaluate the literature on thymic clonal deletion, particularly with an eye to those molecules and processes that have been suggested to have an effect in the late (DP-to-SP transition) deletion models, which are more physiological. Also, because the process occurs as these more mature thymocytes traffic to the medulla, it is helpful to consider what is special about the medullary environment that might regulate this process.

The medulla is specialized for central tolerance

Positive selection occurs in the thymic cortex, where DP thymocytes interact with cortical thymic epithelial cells (cTECs)²⁴. Triggering of the TCR at this stage leads to maturation into SP thymocytes and to the coordinate upregulation of expression of CC-chemokine receptor 7 (CCR7) and other molecules that direct the progenitors towards the medulla^{25,26}. After arriving in the medulla, thymic progenitors interact with different cells than they do in the cortex. For example, the medulla has epithelial cells (medullary thymic epithelial cells, mTECs) that are distinct from those found in the cortex, both in terms of their physical organization and their gene expression. The medulla is also the main site where dendritic cells (DCs) are found in the thymus (FIG. 4).

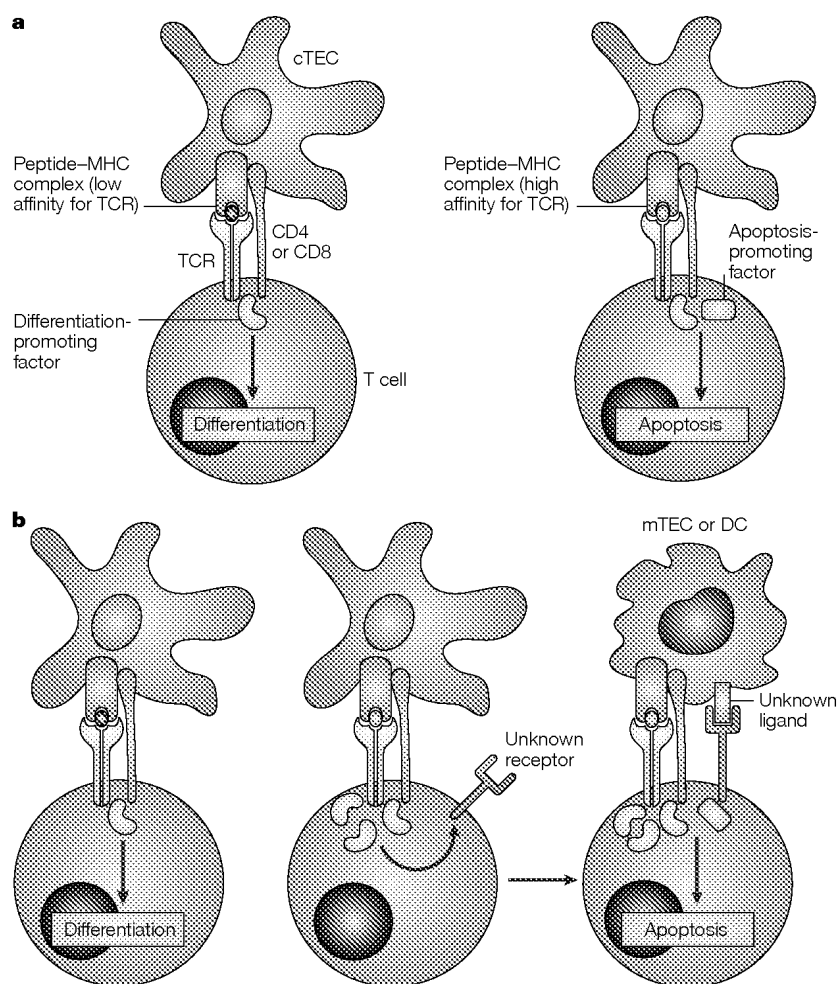


Figure 3 | Models of how differences in affinity affect whether differentiation or apoptosis occurs during development. **a** | The differential-signalling model states that differentiation and cell death are outcomes of unique signals that are delivered by the T-cell receptor (TCR)–co-receptor complex after engagement of low-affinity or high-affinity ligands, respectively, at the surface of cortical thymic epithelial cells (cTECs). This model predicts that the TCR signal is the exclusive difference that drives these markedly different outcomes. **b** | The microenvironmental-cues model states that engagement of high-affinity ligands triggers upregulation of expression of a molecule(s) that provides an additional stimulus for the induction of cell death. Low-affinity ligands might not trigger such changes because the signal that results falls below the threshold of activation for that gene(s). DC, dendritic cell; mTEC, medullary thymic epithelial cell.

The importance of the medulla for central tolerance is underscored by the autoimmune disease observed in various mutant mouse strains that have impaired medullary characteristics: REL-B-deficient mice^{27,28}; *Nik^{aly}/Nik^{aly}* mice, which carry the alymphoplasia mutation in *Nik* (nuclear factor- κ B (NF- κ B)-inducing kinase)²⁹; lymphotoxin- β receptor (LT- β R)-deficient mice^{30,31}; and TRAF6 (tumour-necrosis factor (TNF)-receptor-associated factor 6)-deficient mice³² (TABLE 1; FIG. 4). Mice with these mutations have a disorganized cortico–medullary pattern, generally with a small medulla that lacks mTECs that can be identified with the lectin UEA1 (*Ulex europaeus* agglutinin 1). Positive selection seems to be normal in such mice, but central tolerance is considerably impaired, which results in severe inflammation and/or autoimmune disease. Clonal deletion of endogenous-superantigen-reactive thymocytes is also impaired in REL-B-deficient mice^{33,34}. In addition, the development of NKT cells³⁵ and T_{Reg} cells^{29,32} is also affected in some of these mutant mouse strains (TABLE 1). So, it would seem that both ‘arms’ of central tolerance — clonal deletion, and positive selection of regulatory-cell populations — are affected by these mutations. It is important to note, however, that the phenotypes of these mutant mice are not simple: REL-B, NIK, TRAF6 and LT- β R all have multiple effects on haematopoietic cells, as well as non-haematopoietic cells such as stromal cells. For example, the function of DCs is affected by deficiency in REL-B^{27,28} or TRAF6 (REF. 36), and LT- β R is required for peripheral lymphoid organogenesis³⁷. Nonetheless, analysis of bone-marrow radiation chimeras and recipients of T-cell-depleted thymic grafts showed that the genes encoding these factors have an essential role in thymic stromal elements (that is, mTECs), both for cortico–medullary organization and for thymic selection and autoimmunity (TABLE 1). So, these mutations point to a crucial function for medullary cells in central tolerance.

All of these mutant mouse strains show reduced levels of AIRE, which promotes the expression of peripheral-tissue-specific antigens by mTECs (discussed later). So, a simple hypothesis is that these mutant mice do not display peripheral-tissue-specific antigens in the thymus, and tolerance to these antigens is therefore impaired. Although this might be true, several lines of evidence indicate that it is not the whole story. First, AIRE deficiency alone does not affect the number of T_{Reg} cells^{38,39} or NKT cells, yet T_{Reg} cells and NKT cells are impaired in some of these mutant mouse strains (TABLE 1). The effect on NKT cells and T_{Reg} cells is particularly intriguing, because the self-ligand–MHC complexes that are required for the development of these populations (that is, MHC class II molecules and CD1d, respectively) clearly do not need to be expressed in the medulla^{40,41}. Second, clonal deletion that is mediated by endogenous superantigen is impaired in REL-B-deficient mice, and this superantigen is not expected to depend on AIRE for expression in the thymus. Overall, it would seem therefore that mTECs have an extensive role in promoting central tolerance, which

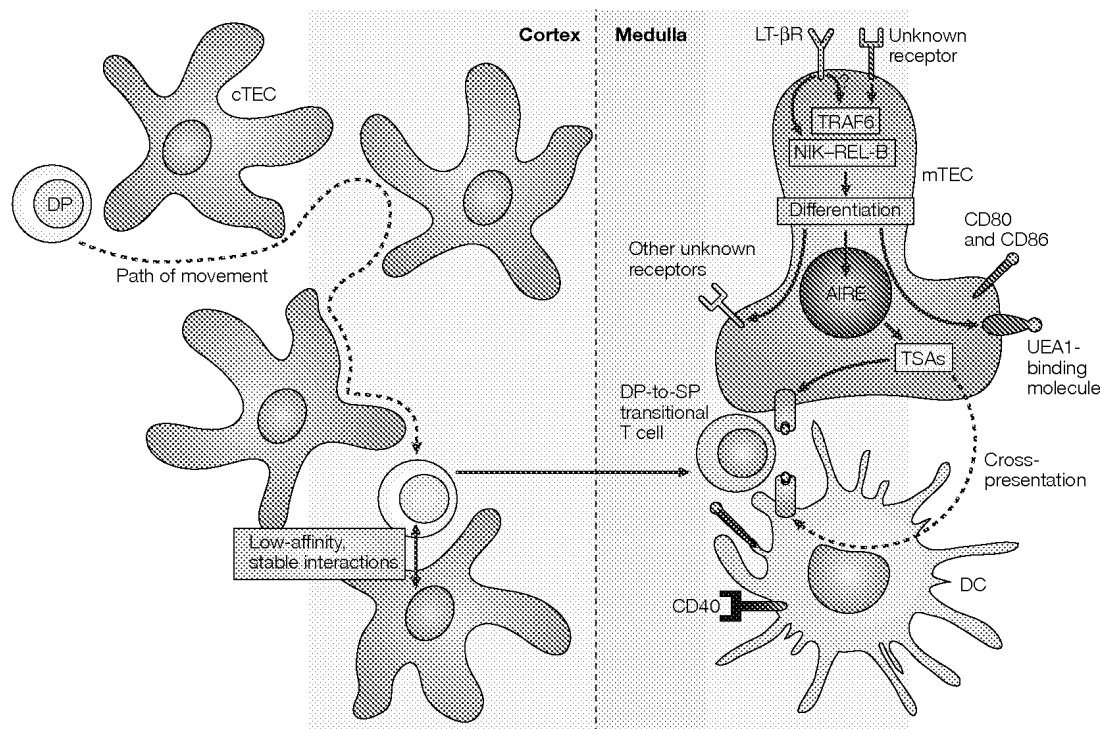


Figure 4 | Aspects of the thymic medulla that are involved in central tolerance. Immature double positive (DP) progenitors rapidly move around the cortex (left panel), until they encounter an appropriate self-peptide–MHC complex on which they can be positively selected²⁴. At this point, their motility decreases, and they undergo a prolonged interaction with the sponge-like cortical thymic epithelial cells (cTECs). After an unknown length of time, they migrate towards the medulla in response to factors that are produced following the interaction of the T-cell receptor with self-peptide–MHC. In the medulla (right panel), these progenitors interact with cells that are distinct from those in the cortex. Medullary thymic epithelial cells (mTECs) have features that might make them specialized for central tolerance. They express co-stimulatory molecules and peripheral tissue-specific antigens (TSAs), some of the genes encoding which are driven by the transcriptional regulator AIRE (autoimmune regulator). Intracellular-signalling molecules that can regulate nuclear factor- κ B (NF- κ B)-family members (such as REL-B) — that is, TRAF6 (tumour-necrosis-factor-receptor-associated factor 6) and NIK (NF- κ B-inducing kinase) — are required for the appropriate differentiation and function of mTECs. Mice with thymic stromal cells that lack these molecules have defects in central tolerance. The lymphotoxin- β receptor (LT- β R) might be involved in the propagation of this NF- κ B-regulating signal or a related signal. Dendritic cells (DCs) are also present in the medulla. They express co-stimulatory molecules, such as CD40, CD80 and CD86. Although DCs do not directly express TSAs, they can cross-present TSAs that are produced by mTECs to developing T cells and thereby induce clonal deletion. SP, single positive; UEA1, *Ulex europaeus* agglutinin 1.

involves more than merely controlling the expression of peripheral-tissue-specific antigens.

In addition to mTECs, DCs also reside in the medulla and are potentially important in the induction of central tolerance. DCs in the peripheral lymphoid organs can CROSS-PRESENT antigens to T cells⁴². But, now, elegant experiments by Gallegos and Bevan⁴³ have shown that thymic DCs can also cross-present antigens — in this case, self-antigens — which results in central tolerance. In this study, they used RIP-OVA mice (which express OVA under the control of RIP, the rat insulin promoter). In these mice, the neo-self-antigen OVA is expressed in the thymus by mTECs. By creating chimeric mice in which the radio-resistant thymus cells (including mTECs) could not present the antigen, they showed that bone-marrow-derived antigen-presenting cells could acquire antigen from mTECs and induce clonal deletion of both developing CD4⁺ and CD8⁺ T cells. Interestingly, they also studied direct presentation by mTECs and found that it did not support deletion of OT-II TCR⁺CD4⁺ T cells, although it did

allow deletion of OT-I TCR⁺CD8⁺ T cells (which are specific for an OVA-derived peptide in the context of H2-K^b) and polyclonal CD8⁺ T cells. Further experiments are needed to determine whether this reflects an inherent difference between developing CD4⁺ and CD8⁺ T cells or is a peculiarity of the OT-II TCR.

Cells of the medulla might contribute to central tolerance through their expression of co-stimulatory molecules, such as CD40, CD80 and CD86. The medulla is the main site of expression of these molecules in the thymus, and they are expressed by both DCs and mTECs. Early on, it was unclear whether co-stimulation mediated by CD80 or CD86 was important for clonal deletion. However, subsequent studies that completely eliminated or neutralized CD80 and CD86 showed that these molecules have a role in the elimination of self-reactive T cells^{44,45}. Similarly, blockade of CD40 prevents the deletion of T cells that is mediated by endogenous superantigen⁴⁶. Interestingly, CD28, CD80 and CD86 are also required for the development of T_{Reg} cells⁴⁷, as are CD40 and CD40 ligand^{38,48}.

CROSS-PRESENTATION

The ability of certain antigen-presenting cells to load peptides that are derived from exogenous antigens onto MHC class I molecules. This property is atypical, because most cells exclusively present peptides from their endogenous proteins on MHC class I molecules. Cross-presentation is essential for the initiation of immune responses to viruses that do not infect antigen-presenting cells.

Table 1 | **Molecules that affect thymic stromal organization and thymocyte selection**

Effect	REL-B*	NIK*	TRAF6*	LT-βR*
On stroma				
Cortico–medullary organization†	Affected§ (REFS 27,28)	Affected§ (REFS 27,28)	Affected§ (REFS 27,28)	Affected§ (REF. 30)
AIRE-expressing mTECs	Strongly reduced (REFS 84,85)	Strongly reduced (REFS 29,30)	Strongly reduced (REF. 32)	Partially reduced (REFS 30,31)
UEA1-binding mTECs	Strongly reduced§ (REF. 28)	Strongly reduced§ (REFS 29,30)	Strongly reduced§ (REF. 32)	Partially reduced§ (REF. 30)
Other medullary effects	Lack CD8 ⁺ DCs (REF. 86); normal CD1d expression (REF. 35)	Strongly reduced ER-TR5 (REFS 29,87) and MTS10 (REF. 30) monoclonal antibody binding§	Normal DC distribution (REF. 32)	Normal DC distribution; partially reduced MTS10 monoclonal antibody binding§ (REF. 30)
On T-cell selection				
Clonal deletion	Impaired for endogenous superantigens (REFS 33,34)	ND	ND	ND
NKT cells	Impaired development§ (REFS 35,88)	Impaired development§ (REF. 87)	ND	ND
T _{Reg} cells	ND	Impaired development (REF. 29)	Impaired development (REF. 32)	Impaired development†
On whole animal				
Disease	Severe inflammation (REF. 27)	Autoimmunity§ (REFS 29,30)	Autoimmunity§ (REF. 32)	Autoimmunity§ (REFS 30,31)

*Mouse strains that lack these molecules show the indicated phenotypic and functional abnormalities. †The defect in cortico–medullary organization is typically a collapsed medullary compartment. §Bone-marrow radiation chimeras or thymic stromal grafting was used to determine that the defect was intrinsic to thymic stroma. ¶Y.-X. Fu, personal communication. AIRE, autoimmune regulator; DC, dendritic cell; LT-βR, lymphotoxin-β receptor; mTEC, medullary thymic epithelial cell; ND, not determined; NIK, nuclear-factor-κB-inducing kinase; NKT cell, natural killer T cell; TRAF6, tumour-necrosis-factor-receptor-associated factor 6; T_{Reg} cell, CD4⁺CD25⁺ regulatory T cell; UEA1, *Ulex europaeus* agglutinin 1.

Because the biochemical mechanisms of apoptosis that operate during clonal deletion are not fully defined, it remains possible that medullary cells express ligands for other receptors that are upregulated by high-affinity TCR signalling in thymocytes. Signalling through these other receptors might determine whether a developing thymocyte undergoes apoptosis or develops into a regulatory T cell. Overall, these data define the medulla as a crucial environment for both aspects of central tolerance: clonal deletion, and positive selection of regulatory T-cell populations.

Molecular regulation of central tolerance

Central tolerance is regulated at several levels. First, only those self-antigens that are presented by thymic antigen-presenting cells can influence central tolerance. So, molecules that regulate the presentation of self-antigens have a crucial influence on tolerance. Second, when progenitors encounter such self-antigens, they can undergo apoptosis or acquire regulatory properties. The transcription factor FOXP3 is important for the differentiation of T_{Reg} cells, and this topic has been reviewed recently⁵. Therefore, our discussion focuses on the molecules that regulate antigen expression and those that are involved in inducing apoptosis.

Mechanisms that regulate tissue-specific-antigen expression in the thymus. An extraordinary finding that has renewed interest in central tolerance is the discovery that AIRE regulates expression of a subset of tissue-specific antigens in the thymus^{39,49} and that mutations in the gene encoding AIRE underlie a specific human

autoimmune disease, APECED^{50–52}. Recent studies in mice have now shown that clonal deletion is impaired by AIRE deficiency. Liston and colleagues^{52,53} showed that the reduced number of CD4⁺ SP thymocytes in transgenic mice expressing both the 3A9 TCR (which is specific for a hen-egg lysozyme (HEL)-derived peptide presented by I-A^b) and HEL under the control of the RIP was restored to normal in the face of AIRE deficiency; a corresponding increase in the incidence of diabetes was also observed. Similarly, Mathis and colleagues⁸⁹ showed that AIRE deficiency increased the number of CD4⁺ SP and CD8⁺ SP thymocytes in RIP–OVA mice co-expressing either the OT-II TCR or the OT-I TCR, respectively, and that diabetes was induced in the OT-I TCR⁺ mice. These findings seem to explain why AIRE-deficient mice have a higher number of activated T cells than do AIRE-sufficient mice and why humans and animals with mutations in the gene encoding AIRE experience autoimmune disease. One question is whether the selection of antigen-specific regulatory T cells is also impaired by AIRE deficiency. AIRE-deficient mice show no reduction in the number of T_{Reg} cells³⁹, the level of FOXP3 expression of these cells, or the *in vitro* or *in vivo* suppressor activity of these cells⁸⁹. In addition, co-transfer of equal numbers of AIRE-deficient and wild-type stromal cells, or AIRE-deficient and wild-type splenocytes, did not inhibit autoimmunity. These results indicate that AIRE-deficient mice do not develop autoimmunity solely because they lack T_{Reg} cells.

How does AIRE promote the presentation of tissue-specific antigens? Evidence clearly indicates that

it has a role in regulating transcription. AIRE contains a nuclear-localization signal and several potential DNA-binding domains⁵⁴. AIRE deficiency alters gene expression by mTECs³⁹, and AIRE has been shown to increase transcription from the promoter of the gene encoding interferon- β ⁵⁵. In addition, it has been shown to bind the transcriptional cofactor CBP (cyclic-AMP-responsive-element-binding protein (CREB)-binding protein), which regulates transcription of a wide range of genes⁵⁶. Despite these data, precisely how AIRE contributes to promiscuous gene expression by mTECs is still a mystery⁵⁷. Work from Kyewski's laboratory⁵⁸ has shown that the genes encoding the tissue-specific antigens that are expressed by mTECs have no obvious structural commonalities that would explain their coordinate regulation, and these antigens are expressed by a variety of peripheral tissues⁵⁸. Interestingly, the genes encoding these antigens tend to be arranged in chromosomal clusters, indicating an epigenetic regulatory mechanism. Similarly, analysis of AIRE-regulated genes also showed chromosomal clustering^{59,60}. Another intriguing observation is that the PLANT HOMEODOMAIN 1 (PHD1) domain of AIRE can mediate ubiquitylation of substrates *in vitro*⁶¹. This E3-ligase-like activity was not present in some human APECED mutants, indicating that it might have a role in the function of AIRE, although this is controversial⁶². As mentioned earlier, AIRE regulates negative selection and prevents diabetes in OT-I TCR⁺ mice. This is consistent with the finding that presentation of OVA antigens is reduced in AIRE-deficient mice. Surprisingly, however, transcripts encoding OVA were still found in AIRE-deficient TECs⁸⁹. Similarly, AIRE-deficient mice show autoimmunity against the ubiquitous protein α -fodrin, despite the fact that transcripts encoding α -fodrin could still be detected in AIRE-deficient thymi⁹⁰. These findings indicate that AIRE has other tolerance-promoting functions in addition to the transcriptional regulation of tissue-specific genes. In this context, the activity of AIRE as an E3 ligase might be of relevance.

It should be noted that AIRE does not regulate the expression of all tissue-specific antigens that are expressed by mTECs. For example, the liver-specific protein C-reactive protein (CRP) and the pancreas-specific protein GAD67 (glutamic-acid decarboxylase of 67 kDa) are expressed by AIRE-deficient mTECs³⁹. This also implies that other molecules or processes are active in this important mTEC function. Insight into this has come from analysis of mice with aberrant mTEC patterning (discussed earlier). The *Nik^{aly}/Nik^{aly}* mutant mouse strain has fewer AIRE-expressing mTECs than do wild-type mice (as do REL-B-deficient, LT- β R-deficient and TRAF6-deficient mice). However, the expression of AIRE-independent tissue-specific antigens, such as CRP and GAD67, is also affected in *Nik^{aly}/Nik^{aly}* mice, even after normalizing for AIRE expression, indicating that a NIK-mediated process controls both AIRE-dependent and AIRE-independent mechanisms of tissue-specific-antigen expression²⁹. Although it is tempting to think that LT- β R signalling

leads to induction of AIRE expression dependent on the TRAF6–NIK–REL-B cascade, this model is probably too simplistic (FIG. 4). First, LT- β R does not directly bind TRAF6. And second, although mice that lack LT or LT- β R have reduced levels of *Aire* expression³¹, this might be secondary to the reduced number of UEA1-binding mTECs, because the few UEA1-binding mTECs that remain seem to express normal levels of AIRE³⁰. Further studies of mTEC development and signalling are clearly needed, but these are challenging because of the low abundance of these cells in the organ.

Differential signalling in T cells during positive and negative selection. The ultimate molecule that is involved in deletion is the antigen receptor itself. Although signals that are provided by the micro-environment are crucial for deletion, it should be emphasized that the TCR itself must be able to discriminate between low-affinity and high-affinity self-ligands. How this discrimination occurs is still enigmatic. An attractive model that has sustained the hopes of many researchers is a conformational-change model: this model proposes that low-affinity and high-affinity ligands induce distinct conformations of the TCR–CD3 complex, which in turn recruits distinct intracellular substrates and leads to the activation of distinct signalling pathways. Data to support this model, however, have not been forthcoming. The structure of the TCR–CD3 complex has not yet been determined, owing to the technical difficulty of modelling large multiprotein complexes in lipid bilayers. Nonetheless, there is indirect evidence that a conformational change occurs on T-cell activation. This was shown by the intracellular-signalling-independent binding of the adaptor protein NCK (non-catalytic region of tyrosine kinase) to an epitope in CD3 ϵ that is only uncovered after TCR triggering⁶³. Recently, Gil and colleagues⁶⁴ showed that both low affinity (positively selecting) and high affinity (negatively selecting) TCR ligands can induce this conformational change in the TCR–CD3 complex in thymocytes, indicating that conformational change is not a determining factor in the outcome of thymic selection. A more likely model is one of kinetic discrimination in which both classes of ligand initiate TCR signalling but only the high-affinity interactions remain associated long enough to allow the recruitment of additional substrates to the active signalling complex. Unfortunately, the identity of these different substrates is unknown at present, although PROTEOMICS APPROACHES are likely to be informative in the future. In this context, many investigators have been interested in linker for activation of T cells (LAT), a multisubunit adaptor protein that is crucial for TCR signalling. LAT has several tyrosine residues in its intracellular domain, and these provide docking sites for various signalling molecules (for example, phospholipase C- γ 1 (PLC- γ 1), growth-factor-receptor-bound protein 2 (GRB2) and GRB2-related adaptor protein (GADS))

PLANT HOMEODOMAIN (PHD). A motif that is found in numerous eukaryotic proteins, many of which regulate transcription. This domain encodes a zinc-binding site and is probably involved in protein–protein interactions.

PROTEOMICS APPROACH
The large-scale analysis of the proteins that are expressed by a particular cell or tissue. This is usually used to compare cell populations, states of differentiation or responses to stimuli. Proteomics not only includes the identification and quantification of proteins but also includes the study of the post-translational modifications and interactions of proteins.

and could translate kinetic differences in TCR signalling into different outcomes on the basis of the speed of phosphorylation of the different tyrosine residues. However, differential phosphorylation of LAT by low-affinity and high-affinity interactions has not been described. Furthermore, mutation of the tyrosine residue at position 136 (the PLC- γ 1-recruitment site) to a phenylalanine residue impaired both positive and negative selection^{65,66}. In fact, this mutation seems to 'shift' the threshold of TCR signalling, resulting in positive selection in response to high-affinity ligands that would normally result in negative selection and in no selection in response to low-affinity ligands that would normally result in positive selection⁶⁷. Conceptually, this indicates that the discrimination between positive and negative selection either involves other tyrosine residues or occurs downstream of LAT. In theory, kinetic discrimination could occur at the level of individual target genes in the nucleus. So, in the next section, we discuss genes that are differentially induced during positive and negative selection.

Despite there being no clear biochemical evidence for differential activation of signalling pathways in positive and negative selection, genetic evidence indicates that there is a differential dependence on signalling pathways. The role of different mitogen-activated protein kinase (MAPK) pathways (that is, extracellular-signal-regulated kinase (ERK) for positive selection, and JUN amino-terminal kinase (JNK) and p38 for negative selection) has been extensively discussed¹. Nonetheless, all three MAPKs seem to be activated by both low-affinity and high-affinity ligands^{68,69}. This has led to the hypothesis that the magnitude or duration of these signals is crucial, an idea that remains to be experimentally validated. An interesting study published in 2005 identified the NIK-related kinase misshapen-like kinase (MINK) as important for negative selection⁶⁹. McCarty and colleagues⁶⁹ showed that MINK-directed SMALL INTERFERING RNA (siRNA) had no effect on positive selection but that it markedly reduced clonal deletion in several models of negative selection, including endogenous-superantigen-mediated thymocyte deletion. They further showed that MINK interacts with NCK and that it is required for both optimal activation of JNK and induction of the pro-apoptotic factor BIM_{EL} (B-cell lymphoma 2 (BCL-2)-interacting mediator of cell death, extra long). It remains to be determined whether MINK is selectively recruited to high-affinity signalling complexes. (It should be noted that NCK is recruited to both high-affinity and low-affinity signalling complexes.) Furthermore, it was recently shown that the recruitment of NCK to the TCR is not required for endogenous-superantigen-mediated clonal deletion⁷⁰. So, although MINK seems to be an important factor, it is unclear precisely how it is involved in signalling pathways that result in the clonal deletion of thymocytes.

Gene-expression changes in T cells during negative selection. Several studies have uncovered genes that are specifically induced by high-affinity interactions

in the thymus. For example, a decade ago, the orphan steroid receptor NUR77 was identified as being expressed in such a manner⁷¹. Although NUR77-deficient mice do not have a defect in thymic clonal deletion, a related family member, neural orphan receptor 1 (NOR1), is also expressed in the thymus⁷², and a dominant-negative form of NUR77 (which also inhibits other NUR77-family members, including NOR1) blocked thymocyte deletion in some models of negative selection⁷³, indicating that NUR77-family members are important. The expression of a novel inhibitor of NF- κ B was also shown to be induced during deletion of DP cells, at least in response to injection of peptide into TCR-transgenic mice⁷⁴. Overexpression of the gene encoding this inhibitor increased CD3-specific-antibody-induced thymocyte death. There is great interest in the role of NF- κ B in T-cell development and selection, but given that this molecule integrates signals from many receptors and has many target genes, interpreting the phenotype of individual gene deficiencies or mutants might be a complex task.

In 2005, two groups have reported a novel approach to the study of gene induction during clonal deletion^{75,76}. Both capitalized on the fact that the NON-OBESE DIABETIC (NOD) MOUSE STRAIN has a defect in thymic negative selection⁷⁷. This not only allowed them to compare the gene-expression profiles of thymocytes undergoing deletion in NOD mice versus control mice but also allowed them to use a genetic-backcross approach to identify the relevant loci that regulate the process. The Mathis and Benoist group⁷⁵ modelled clonal deletion in organ cultures of BDC2.5-TCR-transgenic mice, which express a TCR that is specific for an unknown islet β -cell antigen. Although the NOD background was found to influence clonal deletion, the effect was more robust when a peptide MIMETOPE was added to trigger deletion. The Goodnow group⁷⁶ studied endogenous clonal deletion in mice expressing both the 3A9 TCR and its cognate HEL-derived antigen. The NOD background had little effect on positive selection, implying that the NOD background does not just have a simple effect on TCR signalling. By contrast, both groups showed that NOD mice had a broad reduction in expression of those genes that were normally induced under conditions of negative selection. This broad effect on gene expression indicates that the effect of the NOD background is upstream in the signalling pathway or is affecting a co-stimulatory pathway that is involved in negative selection but not positive selection. Some of the genes that were affected by the NOD background include those encoding programmed cell death 1 (PD1), T-cell-specific adaptor protein (TSAD), 4-1BB, OX40, glucocorticoid-induced TNF-receptor-related protein (GITR), cytotoxic T-lymphocyte antigen 4 (CTLA4), NUR77 and BIM. Surprisingly, genetic studies showed that the NOD-mouse defect in the deletion of BDC2.5 TCR⁺ thymocytes was dominant⁷⁵, whereas in the 3A9 system it was

SMALL INTERFERING RNA (siRNA). Synthetic double-stranded RNA molecules of 19–23 nucleotides, which are used to 'knockdown' (silence the expression of) a specific gene. This is known as RNA interference and is mediated by the sequence-specific degradation of mRNA.

NON-OBESE DIABETIC MOUSE STRAIN (NOD mouse strain). An inbred mouse strain that spontaneously develops T-cell-mediated autoimmune diabetes.

MIMETOPE
When a natural T-cell epitope is not known, it is possible to screen peptides and define a peptide that mimics the stimulatory activity of a natural epitope. This is known as a mimetope.

INSULIN-DEPENDENT DIABETES SUSCEPTIBILITY LOCI

(*Idd* loci). Regions of genomic DNA that are associated with susceptibility or resistance to diabetes in non-obese diabetic (NOD) mice. They were identified by correlating DNA polymorphism with disease in genetic crosses between NOD mice and non-diabetic mouse strains. Because diabetes is a polygenic autoimmune disease, several susceptibility loci have been defined. The *Idd5* region includes the genes that encode CD28, cytotoxic T-lymphocyte antigen 4 (CTLA4) and the natural resistance-associated macrophage proteins (NRAMPs).

BCL-2 FAMILY

(B-cell-lymphoma-2 family). Mitochondrial proteins that increase or decrease the susceptibility of a cell to apoptosis. When the levels of either of the family members BCL-2 or BCL-X_L are increased, a cell is more resistant to cell death.

recessive⁷⁶, indicating that there are unappreciated differences between the deletion models used. As a consequence, the loci that each group determined to be important for negative selection were distinct: they were chromosomes 1, 2, 7 and 15 in the 3A9 model, and chromosomes 1 and 3 in the BDC2.5 model. It is unclear whether the traits mapping to chromosome 1 are the same in both models, but they are near the INSULIN-DEPENDENT DIABETES SUSCEPTIBILITY 5 (*IDD5*) LOCUS, which has been associated with increased resistance to γ -irradiation-induced thymocyte death⁷⁸. Further studies are needed to understand the molecular details of the defect on the NOD background.

In terms of how a thymocyte actually dies during clonal deletion, evidence points more to BCL-2-FAMILY members than to caspase amplification⁷⁹. Overexpression of the anti-apoptotic protein BCL-2 can inhibit negative selection under certain conditions⁸⁰, as can the loss of the pro-apoptotic family members BIM⁸¹ or BCL-2 antagonist/killer (BAK) and BCL-2-associated X protein (BAX)⁸². Interestingly, BIM is present in DP thymocytes, and its expression, particularly in the BIM_{EL} form, is transcriptionally upregulated when these cells are stimulated with high-affinity ligands⁷⁶. The stability and pro-apoptotic activity of BIM can also be post-translationally regulated by ERK-mediated phosphorylation⁸³. So, BIM might be a key effector molecule, the expression of which is regulated both transcriptionally and post-translationally to keep the right progenitors alive during the gauntlet of thymic selection.

Questions remaining

Despite many years of intense investigation, the question of how a single receptor can trigger multiple fates during lymphocyte development is still largely open. Although genetic studies have pointed to key signalling pathways that are involved in positive and negative selection, precisely how these are differentially activated requires further investigation. Ultimately, the answer to this question might require an understanding of the dynamic structure of the TCR-CD3 complex and a kinetic analysis of the 'proteome' that is generated under each condition.

A key model that is emerging in the field is the understanding that progenitors with high affinity for self-antigens can drive either clonal deletion or development into a regulatory T cell. This could be a stochastic process, in which clonal deletion 'de-bulks' the repertoire of dangerous clones, with a few cells remaining to develop into dominant-acting regulatory cells. Alternatively, deletion and regulatory T-cell selection could be processes that are distinguished by subtle differences in affinity or are instructed depending on the particular antigen-presenting cell. There are surprising commonalities between clonal deletion and the selection of regulatory T cells, including a requirement for co-stimulatory molecules (such as CD40, CD80 and CD86) and dependence on an intact thymic medullary microenvironment. The crucial nature of the thymic medulla in particular begs further investigation to determine how mTECs differentiate and what signals they provide to developing T cells.

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Competing interests statement
The authors declare no competing financial interests.

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